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TITLE: MULTI-RESIDUE DETERMINATION OF Β-AGONISTS IN BOVINE MUSCLE USING DISPERSIVE LIQUID LIQUID MICROEXTRACTION BY +ESI TANDEM MASS SPECTROMETRY

BY

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A Thesis Submitted to the School of Graduate Studies in Fulfilment of Requirements for the Degree of Master of Science in Analytical Chemistry

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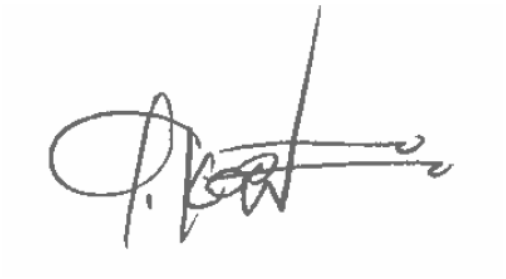
DECLARATION

I hereby declare that the research work submitted for the degree of Master of Science in Analytical Chemistry to the University of South Africa, Pretoria is original except where due reference is made, and was carried out at the Botswana National Veterinary Laboratory between March 2012 and June 2015. This work has not been submitted wholly or in part for the award of a degree or diploma to this or any institution of higher learning.



November 11th, 2017

Signature…… **Date…….**

DEDICATION

I dedicate this work to my father and mother, Mr Solomon Kgothi and Mrs Mokgadi Kgothi. Also to my wife Oratile Mercy Kgothi, my children Antonio Kgothi, Kgotla Amelton Kgothi and Angela Motheo Kgothi.

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I further acknowledge constructive criticism from fellow postgraduate students during periodical meetings held during the course of my studies.

ABSTRACT

A dispersive liquid-liquid microextraction (DLLME) method has been developed, optimized and validated for the extraction of seven beta-agonists (Cimaterol, Cimbuterol, Clenproperol, Clenbuterol, Ractopamine, Isoxsuprine and Ritodrine) from bovine muscle. The homogenized tissue samples were hydrolyzed enzymaticaly by beta-glucuronidase and extracted with DLLME. The extraction parameters (pH, extraction solvent, extraction solvent volume, disperser solvent) were accurately optimized.

Separation of the beta-agonists was by gradient elution on C_{18} LC column using acetonitrile and formic acid aqueous solutions as mobile phases, multiple reaction monitoring (MRM) scan mode was used. The seven beta-agonists were then simultaneous determined and identified in single analysis using 4000 Qtrap LC-MS/MS system. The DLLME method was validated using ISO 17025 and the EU criteria (Commission Decision 2002/657/EC) for validation of analytical method, good precision, repeatability and spiked recoveries were obtained. The limits of detection and quantification for the residues were between 0.0728 – 0.0922 µg/kg and 0.243 – 0.307 µg/kg respectively for beta-agonists. The overall recoveries were between 85% and 100% with the relative standard deviation of (RSDs) between 3.0% and 10%. The recoveries from the developed DLLME method were compared with those obtained from dSPE. DLLME proved to be comparable to SPE. The real samples test showed that the DLLME method developed is accurate and sensitive for the determination of beta-agonist residues in bovine muscle.

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List of Abbreviations

BMC……..…Botswana Meat Commision

BNVL……Botswana National Veterinary Laboratory

SPE……...Solid Phase Extraction

DLLME……..Dispersive Liquid-Liquid Micro-extraction

HF-LPME……... Hollow Fiber Liquid Phase Micro-Extraction

HPLC……..…High Performance Liquid Chromatography

LC-MS/MS……..…Liquid Chromatography Tandem Mass Spectrometry

EU…….……European Union

EC……...European Community

EEC……..…..….European Electoral Commission

LLE……Liquid-Liquid Extraction

GC……..……..Gas Chromatography

AAS……..…….Atomic Absorption Spectrometer

SPME……..…Solid Phase Micro-Extraction

GC-MS……..…Gas Chromatography Mass Spectrometry

RIA……...Radioimmunoassay

ELISA…….……Enzyme-Linked Immunosorbent Assay

MRM…….……Multiple Reaction Monitoring

ESI……..…….Electro-Spray Ionization

DAD…….……..Diode Array Detector

FLD……..……...Fluorescence Detector

MRL……..…Maximum Residue Limit

MRPL……Maximum Permitted Residue Limit

1.4 Declaration of recent publications

Conferences

P. Kgothi, M. M. Nindi and S. Dube. Muliti-residues determination of beta-agonists in bovine muscle using DLLME +ESI-LC-MS/MS, 12th ICCA conference 7-12th July, Pretoria, 2013, South Africa.

P. Kgothi, S. Dube and M. M. Nindi, Dispersive liquid-liquid microextraction of beta-agonist residues from bovine muscle and their determination using electrospray liquid chromatography tandem mass spectrometry, 64th American Society Mass Spectrometry Conference, 3-9th June, San Antonio, 2016, USA.

Manuscripts

P. Kgothi, M. M. Nindi and S. Dube, “Muliti-residues determination of Beta-agonists in bovine muscle using DLLME +ESI-LC-MS/MS”, Manuscript has been submitted for possible publication

CHAPTER ONE

INTRODUCTION

1.1 Background

Beta-agonists are used in human for the treatment of pulmonary disorders. These drugs were also used for this application on meat producing animals before they were banned. When used at higher concentrations in animals, they can act as anabolic steroids promoting muscle development at expense of fat deposition. This ability to suppress fat deposition improves meat quality hence more gain to farmers leading to their abuse. Their use in animal husbandry for exportation to EU mark is prohibited, hence sensitive and reproducible detection techniques are needed to control and monitor their residues in food.

The European Union (EU), United States of America (USA), and some other countries have established Maximum Residue Limits (MRL) and/or Minimum Required Performance Level (MRPL) for veterinary drugs, including beta-agonists. Therefore, several countries have developed sensitive qualitative and quantitative methods for monitoring beta-agonists at trace levels in different matrices. The analytical methods that are employed must be able to detect the beta-agonists residues at, or below, the MRL/MPRL. The Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) and Gas Chromatography Mass Spectrometry (GC-MS) are now commonly used methods as they are most suitable since they comply with the European Union Commission Decision 2002/657/EC, concerning requirements for confirmatory methods. Screening methods such as enzyme-linked immunosorbent assay (ELISA) may show false-positive results due to matrix interference, hence require confirmatory methods. Analytical methods such as

ELISA as well as Gas Chromatography (GC) and Liquid Chromatography (LC) hyphenated to mass spectrometers (GC-MS, LC-MS, LC-MS/MS) have been developed for determination of beta-agonists at MRL/MRPL levels. The MRPL for beta-agonists in bovine muscle has been set at 0.5 µg kg^{-1} by the EU while for animal feed it is 50 µg kg^{-1} [1].

1.2 Analytical methods used for Analysis of beta-agonists

1.2.1 Sample preparation techniques for beta-agonists

In most determinations, sample preparation utilises more than 60% of the total time required for the analysis. Modern analytical chemistry is moving towards miniaturization and simplified analytical procedures. Hence continual development of sample preparation in analytical chemistry is vital. The sample preparation steps in an analytical chemistry, may entail extraction, isolation and enrichment of analytes of interest from a sample matrix. The most commonly used sample preparation techniques for analysis of beta-agonists in various matrices are solid phase extraction (SPE) and liquid liquid extraction (LLE) [2-4]. Dispersive solid phase extraction (dSPE) with various media C2 [5-6], C8 [7-8], C18 [9-11], immunoaffinity [12-16], diatomaceous earth [17-19], ion exchange columns [20-26] have also been used. The major disadvantage of these sample preparation techniques is, the use of significant volumes (milliliters up to liter) of organic solvents. Dispersive liquid-liquid microextraction (DLLME) has been applied for the first time for analysis of beta-agonists in bovine muscle, since it is more economical viable for laboratories with high sample through put, to use less toxic organic solvent (uses microliter volume) and is easy to operate.

1.2.2 Detection techniques used for Analysis of beta-agonists

Analysis of beta-agonists by GC-MS via various derivatization procedures has been reported [2, 27]. Unfortunately, derivatization is a complex and time-consuming procedure. It has been observed that recoveries were adversely affected by incomplete derivatization of the compounds. High-performance liquid chromatography (HPLC) does not require a derivatization step hence can be an alternative to GC-MS analysis. The HPLC technique have been extensively used in quantitative analysis of beta-agonists, using UV [28], fluorescence [29-30], electrochemical [31], and MS [32-35] as detectors. A number of LC-MS/MS multi-residue methods for the determination of up to seven beta-agonists in retina and liver have been previously reported [36-40]. The current procedure determines seven beta-agonists in bovine muscle using enzymatic digestion, LLE, and DLLME, followed by determination of the residues by 4000 Qtrap (+ESI/LC-MS/MS).

1.3 Motivation for the Study

The sample preparation techniques already in use, for Analysis of beta-agonists in various matrices are SPE, dispersive solid phase extraction (dSPE), quick easy cheap effective rugged and safe (QUECHERS) and LLE, use large volumes (in mililiters and above) of toxic organic solvents, which result in disposal being a problem. In laboratories with high sample throughput, these techniques become not economically viable for the routine analysis of beta-agonists.

1.4 Main Objective

The objective of this study was the development of an efficient and economically viable, environmentally friendly, sample preparation (DLLME) method for the analysis of beta-agonists using LC-MS/MS.

Specific Objectives

(1) To develop and optimize LC-MS/MS method for the determination of beta-agonists.

(2) Develop, optimize and validate the dispersive liquid-liquid microextraction (DLLME) method for the extraction of seven beta-agonists.

(3) To apply the validated DLLME-LC-MS/MS method for the determination of beta antagonists in bovine muscle samples.

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

Modern analytical research is oriented towards developing sample preparation methods which are efficient, environmentally friendly and economical. However, the complexity of food samples matrices (matrix interferences) suppress this effort to determine residues of veterinary drugs at trace levels to satisfy food regulations. The wide array of food matrices, from liquids to solids, requires different sample preparation techniques for accurate and reproducible results.

2.1 Origin and chemical properties of beta-agonists

Beta-agonists are alkanolamines (aromatic beta-amino alcohols), compounds which contain both hydroxyl (-OH) and amino (-NH_2, -NHR and –NR_2) functional groups. They constitute a group of compounds such as clenbuterol, terbutaline, salbutamol, fenoterol, salmeterol, ractopamine, bromobuterol, cimaterol, clenpenterol, mabuterol, tulobuterol, mapenterol, cimbuterol, bambuterol, formoterol, orciprenaline, ritodrine, isoxsuprine, labetalol and penbutolol. Beta-agonists are also known as beta-adrenergic agonists [41]. An adrenergic agent is a drug that is capable of either stimulating (agonist) or inhibiting a response (antagonist). This property made them gain popularity in pharmacology, particularly for their anti-inflammatory activities.

2.2 Use of beta-agonists

In humans, beta-agonists are used as bronchodilators by asthmatic sufferers [42-44]. These drugs mainly target the muscles around the airways (bronchi and bronchioles). When these muscles tighten, they make the airways narrower, often leading to suffocation. Administration of beta-agonists relaxes the muscles of the airways resulting in easier breathing. They also have anti-inflammatory activity, which prevent the release and generation of mediators from human lung mast cells that can cause broncho-constriction and inflammation [45]. They can also be abused by athletes as performance-enhancing drugs. Beta-agonists are administered in different ways in humans but the most common way through inhalation [42].

2.3 Health effects of beta-agonists in humans

Side effects of beta-agonists in humans include, increased heart rate and high blood pressure, anxiety, palpitation, as well as skeletal muscle tremors. Also, a case of increased risk of death or near death from asthma associated with regular use of inhaled beta-agonist bronchodilators has been reported [42]. The effects of clenbutarol residues on consumers, especially those who might be taking prescription medication to relieve a pre-existing heart condition, may be detrimental [46]. In the improvement of carcass characteristics in animals and productivity by beta-agonists, the illegal use of beta-agonists has resulted in a number of reports on human food poisoning. For example, in Spain in 2003 [47], Italy in 1997 and 2000 [48-49] and Portugal in 2005 [50], reports of human food poisoning were a result of the ingestion of liver and meat containing clenbuterol residues.

2.4 Legislation and monitoring of beta-agonists in food producing animals

Beef exports to the European Union (EU) have played a major role in economic growth of emerging countries (including Botswana). Regulations are in place to monitor and regulate the import of meat into the European community, which are to be followed. There are measures in place to monitor certain substances (like veterinary medicines) and their residues in live animals and products for safety purposes [51-53]. The use of beta-agonists in veterinary medicine has only been allowed in the case of clenbuterol for bronchodilation in horses and calves, as well as tocolysis in cows [52-54]. The administration of beta-agonists in meat producing animals, as growth promoters, has raised concern in food health, since it leads to high concentration of residues in meat. Beta-agonists fall under group A substances, i.e. prohibited substances in meat producing animals under Council Regulation 2377/90/EC. As a result, no group A substances (beta-agonists inclusive) have been permitted in the European community for growth promoting usage in farm animals [44, 52, 54-56]. There are some countries such as South Africa, Asia and Mexico, which have approved the use of some of the beta-agonists (like Zilpaterol) in cattle as a growth promoter. It becomes a challenge when the neighboring countries are regulating this abuse for EU market purposes [57].

2.5 Sample preparation Techniques

2.5.1 Liquid liquid extraction

Liquid-liquid extraction is an important sample preparation and/or clean-up technique, with a wide range of applications. The extraction process is based on selective partitioning of beta-agonists components versus contaminants in the sample in two immiscible, or partially miscible, liquids. This phenomenon is governed by the distribution coefficient. There are many publications on the determination of beta-agonists using LLE as sample preparation technique [2, 4, 27] and other veterinary drugs residues (steroids and quinolones in food [2, 58]).

2.5.1.1 Drawbacks of liquid-liquid extraction

Liquid-liquid extraction has a number of disadvantages that limit its routine application as a sample preparation technique [2]. These include large solvent volumes (i.e. 20 mL) [2, 4], limited selectivity, difficulty of automation and emulsion formation. This technique works via solubility characteristics of the analytes, and relies on differential solubility of the analytes versus other sample matrix components. Since the solvents used are typically non-polar organics, hydrophobic analytes are extracted into the organic layer, however non-polar interferences are often co-extracted. Not only does this give a less pure extract than desired, but the co-extracts may accumulate on the analytical column, typically a reversed phase column.

2.5.2 Principle of Solid-phase microextraction (SPME)

Solid-phase microextraction (SPME) technique was introduced by Pawliszyn and co-workers in the early 1990s, which allows simultaneous extraction and pre-concentration of analytes from sample matrices [59-62]. It eliminates some disadvantages of conventional techniques such as solid-phase extraction (e.g. plugging of cartridges) and liquid-liquid extraction (e.g. use of toxic solvents) [62]. It is a solvent-less and rapid extraction technique that uses a fused-silica fiber, which is coated on the outside with an appropriate stationary phase. The analytes in the sample (gaseous, liquid and solid) are directly extracted onto the fiber coating. The SPME extraction process are: (1) partitioning of analytes between the extraction

phase and the sample matrix and (2) desorption of concentrated extracts into an analytical instrument [63]. Analyte extraction begins immediately after exposure to the vapour phase above a solution (Headspace-SPME) or direct immersion in the solution (DI-SPME). Once equilibrium is reached between the analyte in the sample and on the fiber (analyte extraction and pre-concentration are combined in single step), the extraction process is complete.

The extracted analytes were then desorbed into a sample vials for chromatographic analysis. The SPME technique can be used routinely in combination with GC-MS, HPLC or LC-MS in different fields. SPME has gained popularity in routine analysis in laboratories and industrial applications in recent years [64-65]. Analysis of food samples [66-70], forensics [71-73], environmental analysis (such as pesticides, phenols, PCBs, PAHs) [74-79] and drug analysis [27, 80]. This technique is been continually developing in terms of new devices, essential parameters in SPME processes like fiber coating and nanotechnology application.

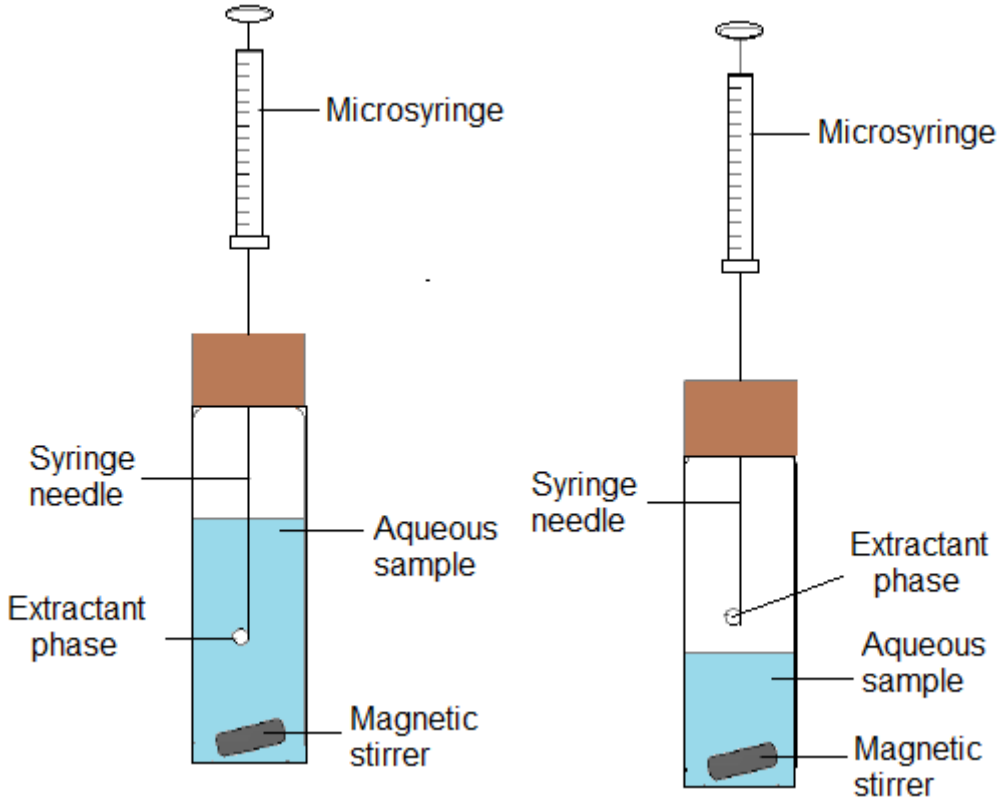
2.5.3 Liquid phase microextraction

LPME is a solvent miniaturized sample preparation procedure of LLE, where a few µL of solvent is used to concentrate analytes from various samples rather than hundreds of mL, needed in traditional LLE. This sample pretreatment technique is compatible with a number of analytical instruments, gas chromatography (GC), capillary electrophoresis (CE) and HPLC. The extraction normally takes place in the acceptor phase (water immiscible solvent), from the donor phase (an aqueous sample containing analytes). Its categories includes, single-drop microextraction (SDME), hollow-fiber microextraction (HF-LPME), and dispersive liquid-liquid microextraction (DLLME).

2.5.3.1 Single drop microextraction (SDME)

SDME uses a microdrop (less than 5 µL) of an organic solvent to extract analytes from the sample solution. After extraction, the microdrop is retracted back into the syringe and transferred for instrumental analysis.

This type of microextraction may be applied to various analytical instruments and analytes with some modifications.



(a) DI-SDME (b) HS-SDME

Figure 2.5: Diagram of a set for direct immersion single drop microextraction (DI-SDME) and headspace single drop microextraction (HS-SDME), (a) and (b) respectively [81].

The HS-SDME follows the same basic principle as traditional SDME, but the drop of organic solvent is suspended in the vapor of a volatile sample in a closed vial. The analytes are extracted from the vapor above the liquid sample. HS-SDME has similar capabilities in terms of precision and speed of analysis as DI-SDME, but has the advantage of a wider variety of solvents to choose from.

The disadvantage of DI-SDME is the instability of the droplet at high stirring speeds. Fast agitation of the sample is employed to facilitate extraction efficiency since agitation allows continuous exposure of the extraction surface area to fresh aqueous sample and reduces the viscosity of the static layer [82].

2.5.3.2 Hollow fiber liquid phase microextraction (HF-LPME)

The HF-LPME was introduced by Pedersen-Bjergaard and Rasmussen [83]. This technique was developed from the basic principle of supported liquid membrane (SLM). Figure 2.6 illustrate the basic principle of HF-LPME.

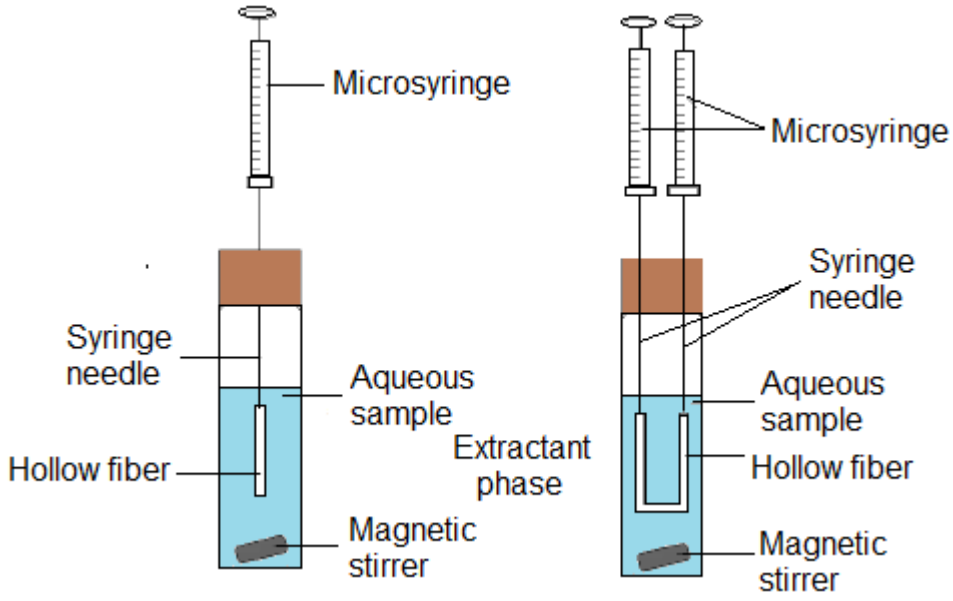


Figure 2.6: A diagram of the structure of a set for hollow fiber liquid phase microextraction (HF-LPME) [82]

The hollow fiber is first washed with acetone in an ultrasonic bath to remove any contaminants and dried in air. It is immersed in the organic solvent for a few minutes to fill its pores, and excess solvent is rinsed off with distilled water. The acceptor phase is carefully injected into the hallow-fiber with a microsyringe.

The HF-LPME can be performed in either 2 or 3 phase modes, whereby in 2 phase mode the acceptor solution is an organic solvent (the same as the one used for the hollow fiber pores). In the two-phase LPME system, the target analytes are extracted

from the aqueous samples and into the organic solvent (acceptor solution) present both in the porous wall and inside the lumen of the hollow fiber. The acceptor solution is then taken for instrumental analysis. In three mode, the acceptor solution may be an acidic or alkaline aqueous solution.

In three-phase LPME, the analytes are extracted from the aqueous sample, through the organic solvent in hollow fiber pores, and further into the aqueous acceptor solution present inside the lumen of the hollow fiber. After extraction the aqueous acceptor solution is taken for instrumental analysis. The first report on HF-LPME described the use of three-phase extraction, with methamphetamine as a model drug [83], while King et al. used two-phase HF-LPME for extraction of polyaromatic hydrocarbons from soil [84].

2.5.3.3 Principle of dispersive liquid liquid microextraction

Dispersive liquid-liquid microextraction is a ternary component solvent system. This method is based on:

(1) An extraction solvent which is mixed with a dispersive solvent. The resulting solvent mixture is then rapidly injected into the aqueous sample.

(2) The rapid injection of the extraction-dispersive solvent mixture produces a cloudy solution containing fine droplets (the analyte in the sample is extracted into the fine droplets) of extraction solvent fully dispersed in aqueous phase,

(3) The analyte has to be in its neutral form for it to be extracted from the aqueous medium (sample) into the organic extraction solvent efficiently. Hence, for the case of beta agonists which exist as polar compounds with pK values greater than one, they need to be neutralized, by optimizing the pH of sample solution, in relation to their pK values.

(4) The cloudy solution is then centrifuged, which causes the extraction phase to sediment at the bottom of the extraction tube, since the extraction phase solvent is heavier than water. The enriched analytes in the sedimented phase are determined by either chromatographic or spectrometric methods.

DLLME has special requirements that must be fulfilled by the extracting solvent and disperser solvents. The requirements for an extraction solvent are: (i) it must be immiscible with water and miscible in the dispersive solvent (ii) must have high extraction efficiency and selectivity for the target analyte (these analytes have to be in neutral form), (iii) must be more dense than water, (vi) must be compatible with the analytical instrumentation being used e.g. GC, GC-MS, HPLC or LC-MS/MS. (v) the volume of extraction solvent is very important, since variation affects the extraction efficiency. An increase in its volume, increases the volume of the sedimented phase, resulting in a decrease of the enrichment factor [85]. Figure 2.7 illustrate the basic principle of DLLME.

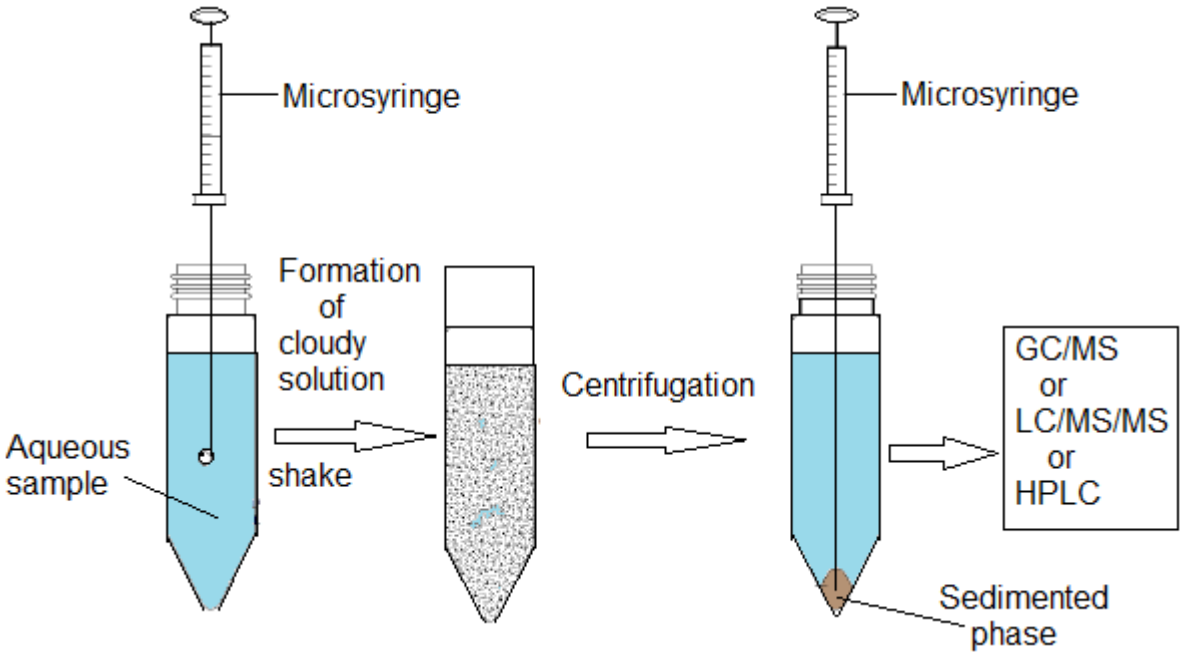


Figure 2.7: The schematic diagram for DLLME process.

The requirements for disperser solvent are: (i) the miscibility of disperser solvent in aqueous phase (sample) and organic phase (extraction solvent) is important. (ii) the volume of the disperser solvent plays an important role, due to the fact that its

variation causes changes in the sedimented phase volume. At low volume of disperser solvent, the dispersion of the extraction solvent and cloudy solution, are not formed completely, while at high volumes, the solubility of analytes in water increases, therefore, the extraction efficiency decreases [85]. The application of dispersive liquid-liquid microextraction (DLLME) on aqueous samples for various organic compounds (including veterinary drugs) has already been done [55, 82-83, 86-98] and tried in solid samples [99]. In this study it has applied to solid matrix (bovine muscle) for determination of beta-agonists.

2.5.3.3.1 Comparison of DLLME with other microextraction techniques

The DLLME, unlike SPME, does not need preconditioning, where the fiber must be pretreated for efficient extraction. The volume of the extractant microdrop in SDME is small – often not bigger than 5 µL, which confines the amount of analytes extracted and the extraction efficiency. DLLME is better since the extraction solvent volume is not limiting. Furthermore, the microdrop in SDME is unstable and easily dislodged from the tip of the microsyringe needle during stirring. Unlike in DLLME, the kind of extractant available for DI-SDME is limited, since it must satisfy certain conditions i.e. water immiscibility, appropriate density. Compared to HF-LPME, DLLME has very short extraction times, mainly because of the large surface area between the solvents (disperser and extractor) and the aqueous phase.

2.5.3.3.2 DLLME and its application

DLLME can be combined with other sample preparation techniques [88]. In some of the work in which matrices other than water were analyzed, the DLLME procedure acted more as a cleaning step than as a pre-concentration procedure. When extracting complex solid samples, the eluate of an organic solvent that has been used to extract the analytes from a solid matrix [98,100-107] or from solid-phase extraction (SPE) containing the analytes [96-97, 108-110] that act as the disperser solvent in the DLLME system. It is from this basis (pre-treatement of solid samples before DLLME) that this study was developed.

The objective of sample preparation is often not only to isolate and concentrate the target analytes (beta-agonists) from the samples (solid i.e. bovine muscle), but also to lower, or even, phase-out the interferences originally present in the sample. The interferences from matrix co-extractives components are often present in DLLME extracts, since it is not a selective extraction method. This is common when determining trace analytes in complex matrices (e.g. bovine muscle). This is the main reason behind reported applications of DLLME being solely focused on simple water samples, so exploration of potential applications of DLLME, with other sample preparation techniques, in more complex matrix samples is the main interest of this study.

Sample pre-treatment before the application of DLLME can be used to improve the selectivity and to improve the limits of quantification (LOQ) in the analysis of complex matrices like food and wastewater. The combination of SPE (as sample pre-treatment) with DLLME, for the selective determination of chlorophenols in aqueous samples with various matrices, has been reported by Assadi et al [111].

2.5.3.3.3 Instruments coupled with DLLME

As a novel sample preparation method, DLLME can be coupled with CE, GC, HPLC, AAS and LC-MS/MS [87, 96]. It has been widely applied to the determination of organic compounds [95, 112] and heavy metals [113].

2.6 Analytical methods for the determination of beta-agonists

The development of radioimmunoassay (RIA) as an extremely sensitive technique for the detection and quantification of very small concentrations of endogenous compounds in biological matrices, caused a revolution in clinical chemistry. Since then, this analytical method has made its way into other disciplines, including veterinary drug residue (like beta-agonists) analysis [114]. Enzyme-linked immunosorbent assays (ELISA) encompass all solid-phase immunoassays using enzyme-labeled reagents. Three versions are commonly used in food analysis: (1) direct competitive ELISA, (2) indirect ELISA, and (3) double-antibody or sandwich ELISA. All these techniques have been used for the determination of beta-agonists residues in animals [114]. However, there are some drawbacks associated with ELISA, due to interferences in complex biomatrices such as meat, which frequently lead to low recovery and false-positive/negative results. There is therefore a need to confirm the results with more precise techniques, like HPLC, GC and LC-MS/MS [57,

115-116]. Furthermore, there are instances of cross-reactivities (undesired side-reactions) [67]. ELISA also has some limitation in multi-compound determinations, since it is compound specific [117]. The use of HPLC with different detectors, GC-MS and LC-MS/MS for determination of beta-agonists has been reported [116].

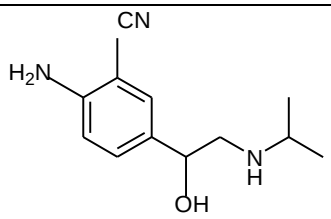
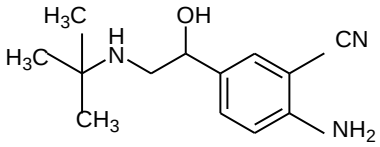
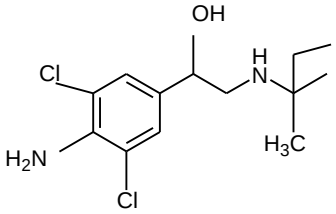
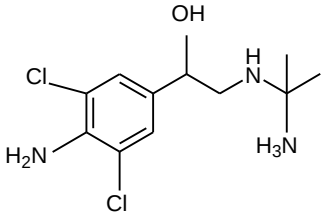
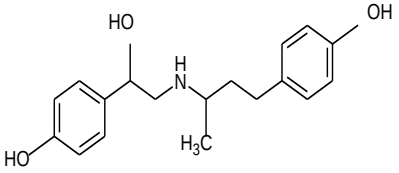
CHAPTER THREE

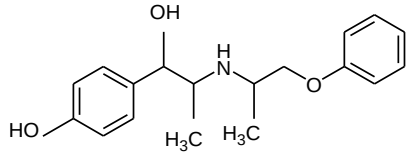
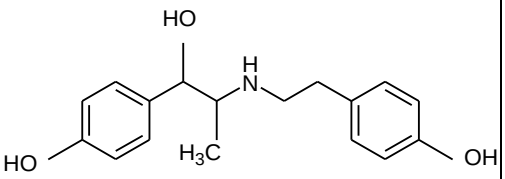
3. EXPERIMENTAL

3.1 Material and Reagents

HPLC grade acetone, ethanol, methanol, dichloromethane, chloroform, trichloromethane, tetrachloroethylene, formic acid and 2-propanol (Merck, Darmstadt, Germany) were used in this study. LC-MS grade acetonitrile, sodium acetate and ammonium formate (analytical reagent grade) were obtained from Sigma-Aldrich (Munich, Germany). Ultra high purity (UHP) water, with a resistivity of 18.2 $M\Omega cm^{-1}$, was obtained using a Milli-Q system Millipore Corp., (Molsheim, France). The following standards; ritodrine chloride (95.5%), isoxsuprine chloride (99.1%), clenbuterol chloride (95.5%), ractopamine chloride (95.5%), cimaterol (99.8%), cimbuterol (99.9%), clenproperol (99.9%) and β-glucuronidas were obtained from Sigma-Aldrich (Munich, Germany). Table 3.1 summarizes the chemical structures, m/z, pKa values (1&2) and CAS numbers of all the beta-agonists in this study.

Table 3.1 Name, chemical structure, physical properties and CAS numbers of all the beta-agonists in this study.

Name	Structure	pKa_1	pKa_2	CAS number
Cimaterol	 *m/z* = 220	13.75	9.24	54239-37-1
Cimbuterol	 *m/z* = 234			
Clenproperol	 *m/z* = 263			38339-11-6
Clenbuterol	 *m/z* = 278	9.63	14.06	37148-27-9
Ractopamine	 *m/z* = 302	9.4		97825-25-7

Name	Structure	pKa_1	pKa_2	CAS number
Isoxsuprine	 *m/z* = 302	8	9.8	0000395-28-8
Ritodrine	 *m/z* = 288	9.15	9.81	26652-09-5

3.2 Sampling and storage of samples

The bovine muscle samples used in this work were sampled from two Botswana Meat Commission (BMC) abattoirs, at Lobatse (South region) and Francistown (North region), as well as in different district municipal abattoirs. Sampling was performed in accordance with the Commission Decision 98/179/EC of February 1998 [123] and Directive 2002/63/EC or superseding legislation. The fresh samples were packaged in polythene bags, placed in cooler boxes with ice packs and transported to the laboratory within 24 hours. The samples were assessed upon arrival in the laboratory, to check if they were fit for laboratory testing, then passed on to the residues testing unit. The bovine muscle samples used in this study, were cut into smaller portions before being frozen (at ≤ - 20 °C) to avoid thawing and cutting of samples which compromises sample integrity.

3.3 Instrumentation

The LC-MS/MS system consisted of an Agilent 1200 HPLC system from Agilent Technologies (Waldbronn, Germany) and an Applied Bio-systems 4000 Qtrap mass spectrometer (Darmstadt, Germany) with Turbo V^{TM} Ion source mass spectrometer (MS/MS). The Agilent 1200 HPLC system consisted of an auto-sampler, a quaternary pump and a thermostated column compartment. Analyst 1.5.2 software was used for instrument control and data processing.

Precisa 220M (Lancashire, United Kingdom) and Adam PGM 253e (Bletchley, United Kingdom) analytical balances were used for weighing the reagents and samples that were used in the experiments. RADWAG AS 220/c/2 Supermicro balance (Bracka, Poland) was used to weigh analytical standards during standards preparation. The samples were centrifuged in a Herus Biofuge PrimoR centrifuge Thermo Electron Cooperation (Steingrund, German). Mettler Toledo (seven Multi) pH meter (Greifensee, Switzerland) was used for pH measurements. TurboVap (Turbo vap LV) Casper Life Sciences Automatic concentrator (Hopkinton Mass, United State of America) was used for evaporating the solvents during sample preparation.

3.4 Standard solutions

Standard solutions of cimaterol, cimbuterol, clenbuterol, clemproperol, isoxsuprine, ractopamine and ritodrine were individually prepared, by dissolving 10 mg in 10 mL of methanol, to make a 1 mg mL^{-1} stock solution, which was then stored at -20 °C. All standards were prepared by taking into consideration the percentage purity of standard and salt content. The intermediate standards and working standards were prepared from the stock standards. The intermediate and working standards were stored in a fridge (2 °C to 6 °C).

3.4.1 Preparation of calibration and spiking solutions

The standard solutions for the calibration curve and spiking experiments were prepared in methanol. The calibration standards were made by serial dilution of the stock solution and the calibration curves were calculated using the linear regression model. The spiking levels were 0.5, 0.75 and 1.0 µg kg^{-1}.

3.5 Method development MS/MS conditions

A 1.0 µg mL^{-1} standard mixture of seven Beta-agonists standards was diluted 100 times by drawing up 100 µL into the syringe, then emptying the syringe followed by filling the syringe with 1 mL of methanol (0.1% formic acid). This mixture was infused at 10 µL min^{-1}, using an infusion pump (11 plus) ABI into mass spectrometer with Q1 scan (to confirm the presence of compounds of interest). Having entered the calculated mass of each analyte, with a width of 50 Da, a peak at $[M + H]^+$ was obtained. Compound optimization for each beta-agonist compound was run after observing a peak at $[M + H]^+$. The MS optimizes the ion path for the beta-agonists compounds by ramping the ion optic voltages from low to high and determining the optimum value. It also determines the most intense fragments, with their respective collision energies and creates an MRM method. 0.01 µg mL^{-1} of seven Beta-agonists standard mixture was used for optimization of the ion source and gas parameters (Gases, IonSprayTH Voltage & temperature) and MRM scan mode was used.

3.5.1 Ion source optimization parameters for beta-agonists

The ion source parameters to be optimized are given in Table 3.51. It is of paramount importance to optimize the ion source parameters for mass spectrometry analysis, for the method to be selective and specific. The standard mixture (10 µg L^{-1}) of beta-agonists was infused (using syringe pump with flow rate of 100 µL min^{-1}) in to the mass spectrometer with Q1 scan to obtain $[M + H]^+$.

Table 3.5.1: The ion source parameters to be optimized

Ion source parameters	
Ionspray™ Voltage (IS)	Collision Gas (CAD)
Curtain Gas™ (CUR)	Declustering Potential (DP)
Temperature (TEM)	Entrance Potential (EP)
Ion Source Gas 1(GS1)	Collision Energy (CE)
Ion Source Gas 2(GS2)	Collision Cell Exit Potential (CXP)

3.5.2 Optimization of compound dependent parameters for beta-agonists

The following compound dependent parameters are to be optimized: a. Declustering Potential (DP), b. Entrance Potential (EP), c. Collision Energy (CE), d. Collision Cell Exit Potential (CXP).

It is important that when developing a method with mass spectrometry analysis, the precursor ion, $[M+H]^+$ when using positive ionization mode or $[M-H]^-$ when using negative ionization mode is observed before optimizing the compound dependant parameters of the mass spectrometer. This is to assure that a proper molecule is detected. In this instance, all the precursor ions for the seven beta-agonists studied were the predominant peaks in the spectra observed after infusion. This was achieved by Q1 scan.

3.5.2.1 Multiple Reaction Monitoring (MRM) scan

This scan type was performed during compound optimization parameters of the mass spectrometer. Moderate collision energy (52 volts) was used hence precursor ions were observed, for instance in the cimaterol case, the 220.2 ion was optimum.

3.5.2.2 The product Ion scan of cimaterol

The product ion scan is an MS/MS scan, where the first Quadrupole (Q1) is fixed and the third Quadrupole (Q3) sweeps a range. It is an experimental tool used to search for all products of a particular precursor ion (instant 220.2 ion). The collision energy of the Q2, which is a collision cell, was ramped incrementally, from 52 volts to 92 volts. At low CE values, high molecular weight (MW) product ions are formed and most of the precursor ion are kept intact, whereas increasing the CE to higher values, low MW product ions are formed.

3.6 HPLC separation of seven beta-agonists

A phenomenex Luna Kinetex (150 x 3 mm I.D., 5 µm particle size) analytical column (Aschaffenburg, Germany) was used at constant temperatures of 30 °C and the sample injection volume was 10 µL. Mobile phase A consisted of 0.1% v/v formic acid at pH 3.83, whereas mobile phase B consisted of acetonitrile with 0.1% v/v formic acid at pH 3.56 (50:50, v/v). The pH of the formic acid was adjusted with 40 mMol ammonium formate buffer. The flow rate of the mobile phase was 0.25 mL min^{-1}. A gradient elution method was used with solvent A starting at 80% and held for 2 min, then dropped to 60% in a minute and held for 2 min. The mobile phase composition was then raised to 80% solvent B in 3 min and maintained for 2 min before next injection.

3.7. Sample preparation before DLLME procedure

The homogenized muscle samples (2.0 ± 0.02 g) were placed in 50 mL polypropylene centrifuge tubes and spiked with 0.5, 0.75, 1.0 µg kg^{-1} of the standard solution mixture of containing seven beta-agonists. A 10 mL aliquot of 0.2 M acetate

buffer, at pH 5.00, was added to the samples and gently mixing, then vortexed for 4 minutes. A 40 µL aliquot of β-glucuronidase was then added, for enzymatic hydrolysis, before incubation for 2h at 40 °C for 2h. The supernatant was decanted and placed in another container, where 0.8 g sodium chloride, 0.4 g sodium sulphate and 0.2 g magnesium sulphate, were added. The sample was then centrifuged at 4000 rpm for 10 min. The resulting supernatant was filtered using 0.45 µm nylon filters and the pH adjusted to 11 with 40 mMol ammonium formate.

3.7.1 DLLME procedure

For DLLME, the pH, disperser solvent, extraction solvent and the volumes of both the disperser and extraction solvents were optimized.

3.7.1.1 pH optimization

The extraction of the analytes was investigated between pH 8 and 12 since the pKa values of the compounds under study were within this range. Using a 5 mL supernatant aliquot of sample, 1mL of 2-propanol (as disperser solvent) and 100 µL of chloroform (as extractor solvent), were used as the standard sample volume to optimize pH.

3.7.1.2 Selection of disperser solvent

Ethanol, methanol, acetonitrile, acetone and 2-propanol were investigated as possible disperser solvents. The extraction conditions used in this study were; 5 mL supernatant aliquot at pH 11, 1 mL of disperser solvent and 100 µL of the extraction solvent.

3.7.1.3 Optimization of disperser solvent volume

The effect of the volume of disperser solvent on the extraction efficiency was studied using 5 mL supernatant aliquat at pH 11, while varying the volume of disperser solvent (500-2500 µL) and 100 µL of extractor solvent.

3.7.1.4 Selection of extraction solvent

Four extraction solvents; chloroform, dichloromethane, tetrachloroethylene and trichloromethane were investigated. A 5 mL of sample supernatant at pH 11 and 1 mL of disperser solvent, while varying the volume of extractor solvent, were used in the experiments.

3.7.1.5 Optimization of extraction solvent volume

Different volumes of extraction solvent (dichloromethane), in a range of 50-200 µL were investigated. The extraction conditions, 5 mL supernatant aliquot at pH 11, 1 mL of acetonitrile disperser solvent, were used.

The DLLME procedure was, therefore, as follows: 1 mL of acetonitrile (disperser solvent) and 100 µL of dichloromethane (extraction solvent) were immediately injected into the 5 ml supernatant and thoroughly shaken to give a cloudy mixture. The mixture was then centrifuged and the sedimented solution was transferred into vials for LC-MS/MS analysis.

3.7.2 Validation of the optimized DLLME method

The optimized DLLME method was validated according to the Commission Decision 2002/657/EC and ISO 17025 [104]. The limit of detection (LOD), limit of quantification (LOQ), correlation coefficient (R^2), accuracy and relative standard deviation, were used to express repeatability. In addition the decision limit (CC α) and detection capability (CC β), were calculated based on the MRLs of the compounds. The extraction recoveries and extraction efficiencies were also determined.

3.7.2.1 Procedure for linearity

Linearity was determined by spiking blank bovine muscle samples with beta-agonists standard mixture at concentractions corresponding to eight calibration levels in the range of 0.5 – 2.0 µg kg^{-1}. A total of three spiked samples at each spiking level were taken through the entire DLLME procedure and used to construct a calibration curve.

3.8 dSPE procedure

The sample pre-treatment was as above (Section 3.7) up to enzymatic hydrolysis. The supernatant was decanted and placed into a Pyrex tube and 1.2 mL of 1 M HCl was added. A 7 mL aliquot of sample extract was loaded onto a SPE A Strat-X-C 3 mL/60 mg which had been previously conditioned with 2.0 mL of methanol and washed with 2.0 mL 0.1 M hydrochloric acid. After loading the sample, the cartridge was washed with 1.5 mL 0.1 M HCL followed by 1.5 mL of methanol and finally eluted with 3 mL of ammonium hydroxide/methanol (5:95% v/v). The eluate was dried and reconstituted with the mobile phase for LC-MS/MS analysis.

CHAPTER FOUR

4.0 Results and Discussion

4.1.1.1 MS/MS method development

A mass spectrometry method was developed and validated for the determination of seven beta-agonists. It was achieved by optimizing the following parameters of the mass spectrometer: (i) ion source and (ii) compound dependent parameters. Table 4.1.1 shows the ion source parameters for the beta-agonists.

Table 4.1.1: Ion source parameters

Ionspray parameter	Optimized parameter
Ionspray™ Voltage (IS)	4500 volts
Curtain Gas™ (CUR)	25 °C
Temperature (TEM)	600 °C
Ion Source Gas 1(GS1)	40 °C
Ion Source Gas 2(GS2)	40 °C
Collision Gas (CAD)	Medium
Declustering Potential (DP)	16 volts
Entrance Potential (EP)	10 volts
Collision Energy (CE)	25 volts
Collision Cell Exit Potential (CXP)	14 volts

4.1.2 Compound optimization parameters for beta-agonists

In this study, the precursor ions were predominant peaks in all seven beta-agonists of interest. Compound optimization parameters for beta-agonists, obtained from Applied Biosystem API 4000 Qtrap mass spectrometer with Turbo V^{TM} Ion source, LC-MS/MS in +ESI mode and MRM scan type, as shown table 4.1.2. These compound parameters allow the equipment to be very sensitive to detect and quantify these beta-agonists. Deviation from these parameters, for this equipment, will result in a loss in sensitivity.

Table 4.1.2 Compound optimized parameters of beta-agonists in LC-MS/MS

Compound	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	Transitions	DP (volts)	EP (Volts)	CE (Volts)	CXP (volts)
Cimaterol	220.3	160.1	150	Cimaterol T1	16	10	25	14
		143.1	150	Cimaterol T2	16	10	33	10
		116	150	Cimaterol T3	16	10	49	8
Cimbuterol	234.3	160.1	150	Cimbuterol T1	11	10	21	10
		143.1	150	Cimbuterol T2	11	10	37	10
		116.1	150	Cimbuterol T3	11	10	49	8
Clenbuterol	278.2	204	150	Clenbuterol T1	51	10	25	18
		203	150	Clenbuterol T2	51	10	23	16
		133	150	Clenbuterol T3	51	10	39	10
Clenproperol	263.2	132	150	Clenproperol T1	11	10	37	10
		203	150	Clenproperol T2	11	10	27	18
		168.1	150	Clenproperol T3	11	10	39	14
Isoxsuprine	302.3	107	150	Isoxsuprine T1	101	10	39	6

Compound	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	Transitions	DP (volts)	EP (Volts)	CE (Volts)	CXP (volts)
		150.1	150	Isoxsuprine T2	101	10	31	12
		77	150	Isoxsuprine T3	101	10	85	12
Ractopamine	302.3	164.2	150	Ractopamine T1	1	10	23	14
		107.2	150	Ractopamine T2	1	10	45	8
		121.2	150	Ractopamine T3	1	10	33	8
Ritodrine	288.3	121.1	150	Ritodrine T1	96	10	33	8
		150.1	150	Ritodrine T2	96	10	27	12
		77.1	150	Ritodrine T3	96	10	77	4

4.2. LC Method development

4.2.1 Liquid chromatography optimization

A chromatographic separation method was developed for the determination of seven beta-agonists using. Phenomenex Luna Kinetex (150 mm x 3 mm, 5 µm analytical column, at a constant temperature of 30 °C. The optimized chromatographic separation was achieved by changing different mobile phase (A and B) compositions and flow rates, one parameter at a time. Figure 4.2 shows a typical chromatogram obtained for the separation of seven beta-agonists. The linearity, correlation coefficient, limit of detection, limit of quantification, and the reproducibility were investigated.

Figure 4.2.1 shows a chromatogram of formate buffered separation. The seven beta-agonists were well resolved except ractopamine and isoxsuprine, which were co-eluting due to their isomerism (*m/z* of 302.3). The improved LC method was then used to validate the DLLME method.

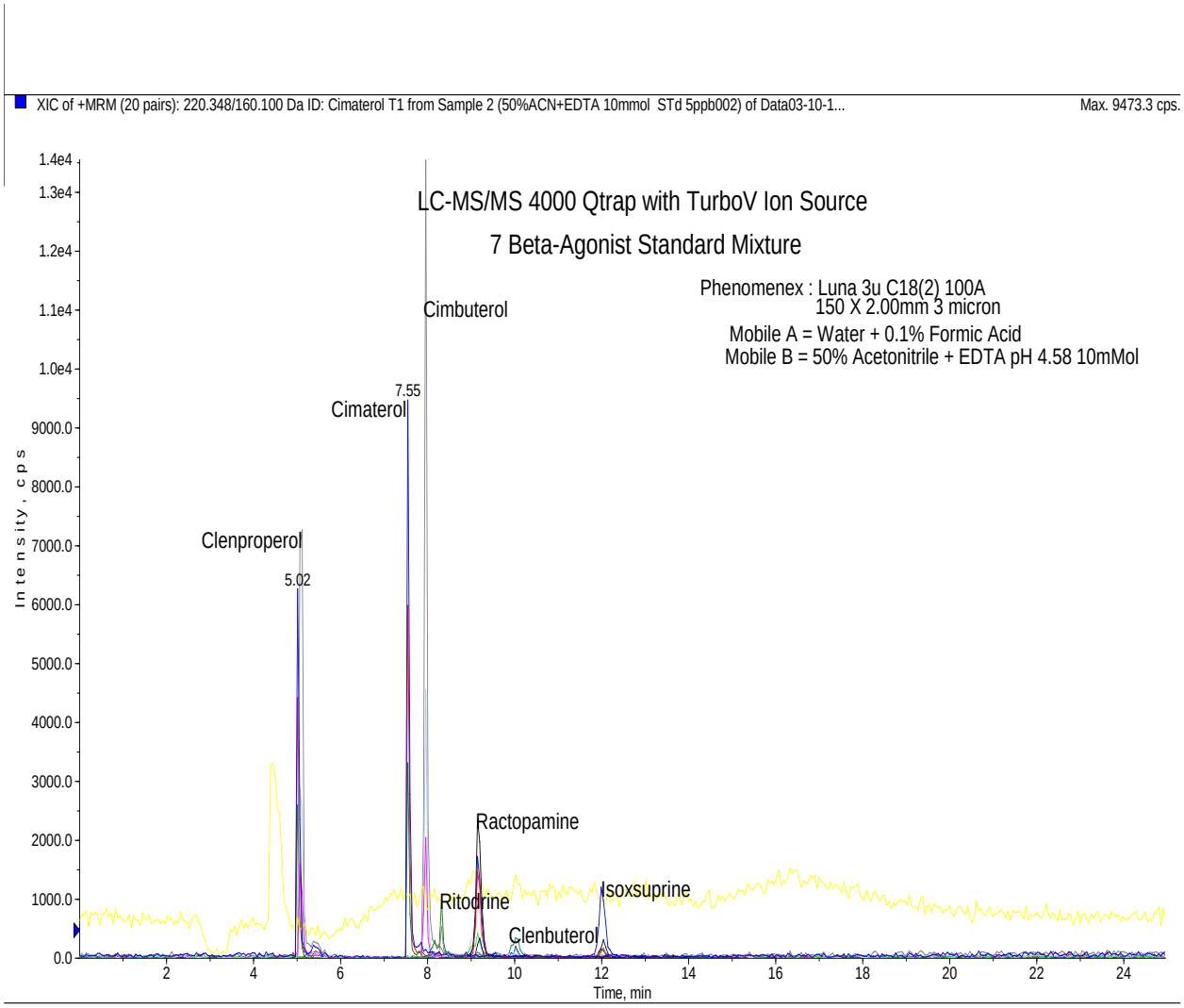


Figure 4.2: Chromatogram of seven beta-agonists at 0.5 ppb concentration.

The above chromatogram was as a result of developed separation method of seven beta-agonists under the study. The developed separation method had some limitations, the ethylenediaminetetraacetic (EDTA) buffer was not suitable (since was not volatile) for LC-MS/MS analysis, was clogging the orifice of the ion source. Also, the above method was less sensitive. The EDTA buffer was then replaced with 40 mM ammonium formate buffer, which is more volatile.

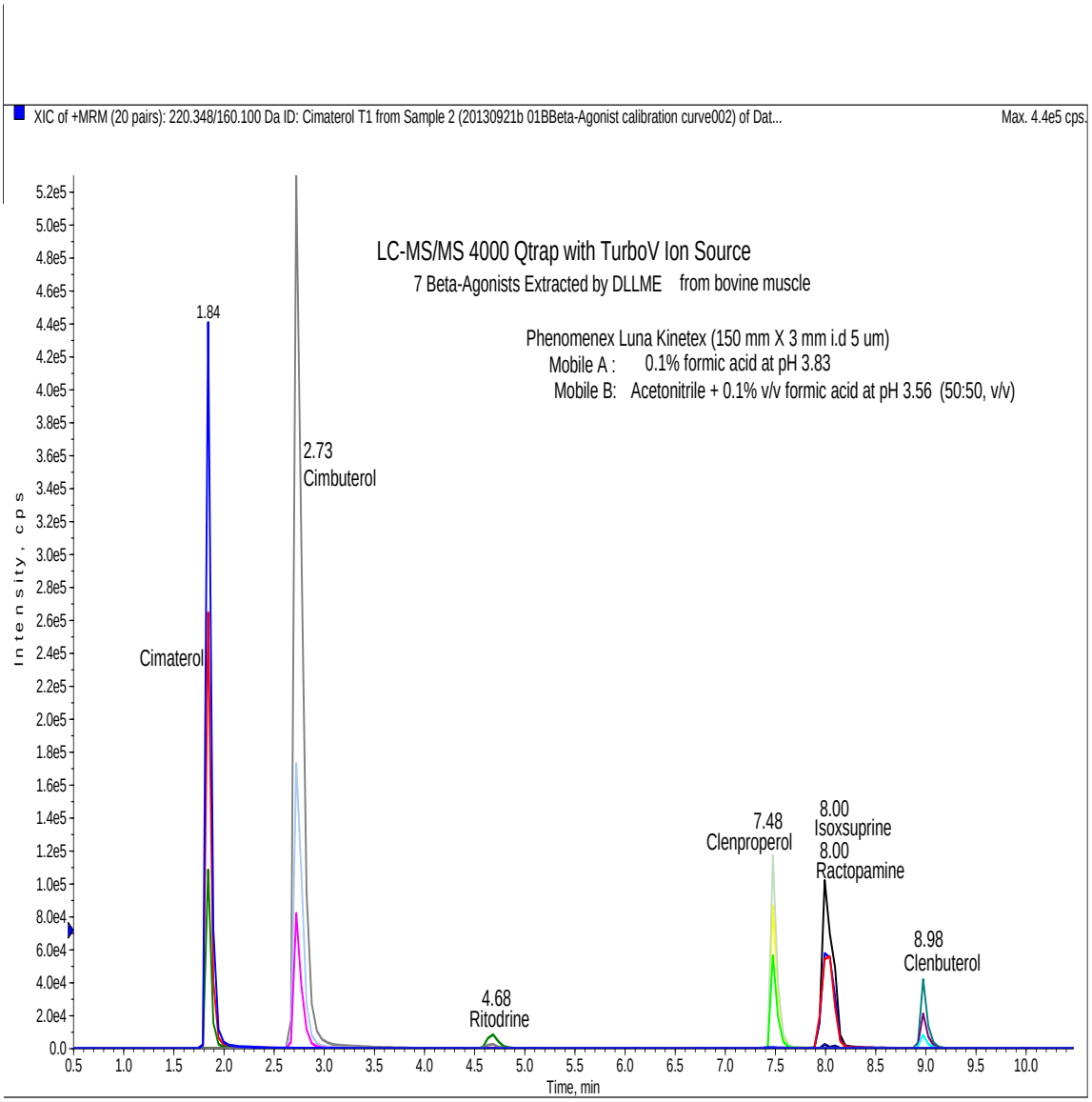


Figure 4.2.1: Separation of seven beta-agonists at 0.5 ppb level using ammonium formate buffer replacing EDTA buffer.

The chromatogram in figure 4.2.1 indicates an improvement of sensitivity, shorter run time and no clogging of the orifice. The seven beta-agonists were well resolved, except ractopamine and isoxsuprine, which were co-eluted, since they are isomeric

(*m/z* of 302.3) compounds. These compounds will still be quantified with ion abstraction using MRM mode, since they have same target ions but different daughter ions. The improved LC method was then used to validate the DLLME method.

4.3 DLLME Method development

Dispersive liquid-liquid micro-extraction was selected as a sample extraction and/or clean-up method for beta-agonists in bovine muscle samples. Extraction of analytes from bovine muscle is usually a challenge due to the presence of fat. The fat was removed by firstly treating the sample with salts and organic solvent prior to application of DLLME treatment. Parameters including extraction solvent, disperser solvent, sample pH as well as volume of extraction and dispersive solvents were optimised.

4.3.1 Selection of extraction solvent

The extraction solvent is one of the most important parameter that needs to be optimized for successful application of DLLME. Four extraction solvents were selected; chloroform, dichloromethane, tetrachloroethylene and trichloromethane for the extraction of beta-agonists. Chloroform (1.48 g mol^{-1} at 20 °C), dichloromethane (1.34 g ml^{-1} at 20 °C), trichloroethane (1.33 g mol^{-1} at 20 °C) and tetrachloroethylene (1.62 g mol^{-1} at 20 °C) were used. Dichloromethane was selected as the ideal solvent, since the extraction efficiencies ranged from 80 - 92% followed by chloroform with 70 – 88%, (Figure 4.3.1). Tetrachloroethylene and trichloromethane demonstrated low extraction efficiencies which were between 30 – 55% and 25 – 48 % respectively.

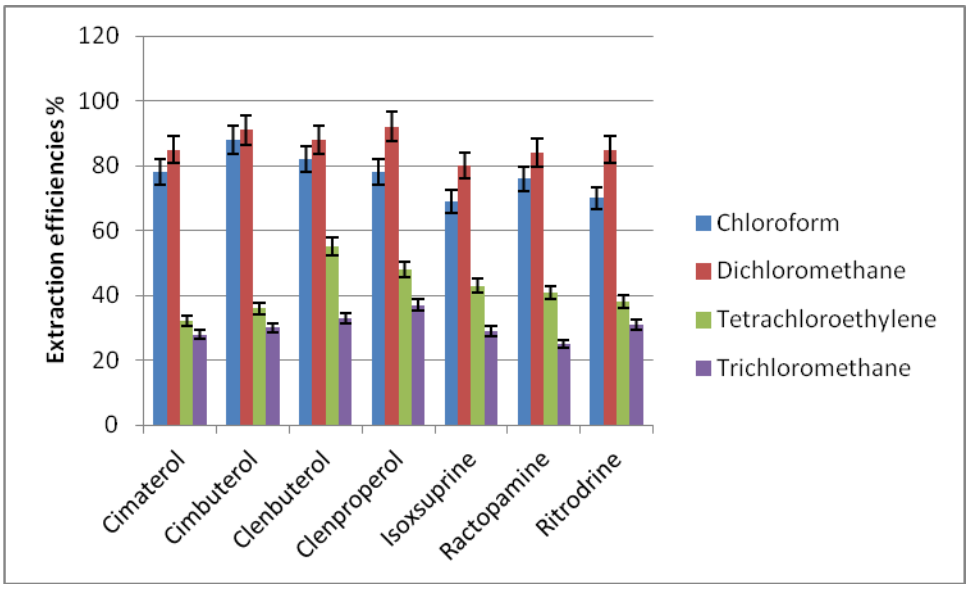


Figure 4.3.1: Selection of extraction solvent for beta-agonists

4.3.2: Selection of dispersive solvent

The selection of the disperser solvent is also an important parameter in developing DLLME method. In this study, five solvents were investigated as possible disperser solvents; 2-propanol, methanol, ethanol, acetonitrile and acetone. Most beta-agonists extracted well with acetonitrile, since the extraction efficiencies ranged from 68 -90 % (Figure 4.3.2). It was also observed that 2-propanol extracted reasonably well as a disperser solvent with extraction efficiencies ranging from 55 – 80%. This disperser solvent (2-propanol) seemed to be the most efficient for isoxsuprine (78%) and cimaterol (79%), while the extraction efficiencies for the same analytes with acetonitrile were isoxsuprine (68%) and cimaterol (77%). Methanol and acetone were not as efficient as disperser solvents with extraction efficiencies lower than 50% for all analytes, this could be due to their less solubility in these solvents. On

the basis of extraction efficiencies, acetonitrile was selected as disperser solvent for the extraction all of the beta-agonists.

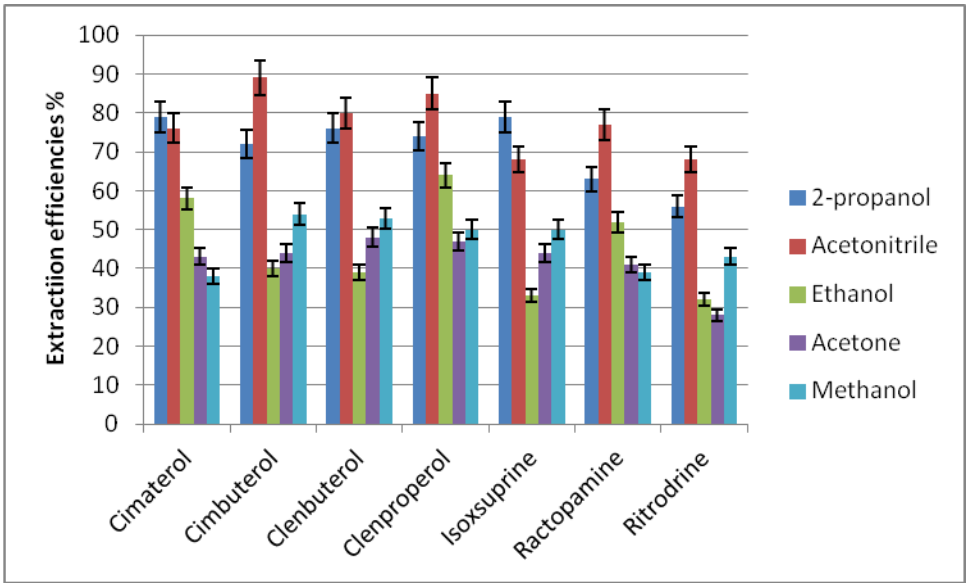


Figure 4.3.2 Selection of disperser solvent**.**

4.3.3 pH optimization of DLLME for beta-agonists

In dispersive liquid liquid micro-extraction, the analyte must be in its neutral form to be extracted from the aqueous phase (supernatant solution) and into the organic extraction solvent efficiently. Beta agonists under study are polar compounds which need to be neutralized by optimizing the pH of sample solution in relative to their pKa values. The partitioning of an analyte from an aqueous phase into a hydrophobic organic solvent, is greater for a neutral compound than for an ionized compound.

The effect of pH on the extractability of beta-agonists was investigated in the range of 8 – 12. Sodium hydroxide and formic acid solution were used to adjust the pH. The extraction efficiency at pH 8, 9, 10, 11 and 12, are illustrated in figure 4.3.3.

Cimaterol, cimbuterol, isoxsuprine and ritrodrine had the highest recoveries at pH 11, while, clenbuterol and clenproperol, at pH 12. The difference in the extraction of clenbuterol and clenproperol at pH 11 and 12 was very minimal, for example clenproperol had extraction efficiency of 90% and 95% at pH 11, 12 respectively. 6 beta-agonist had extraction efficiency above 60% at pH 11, as compared to other pH levels hence pH 11 was selected as optimum pH for the extraction of beta-agonists.

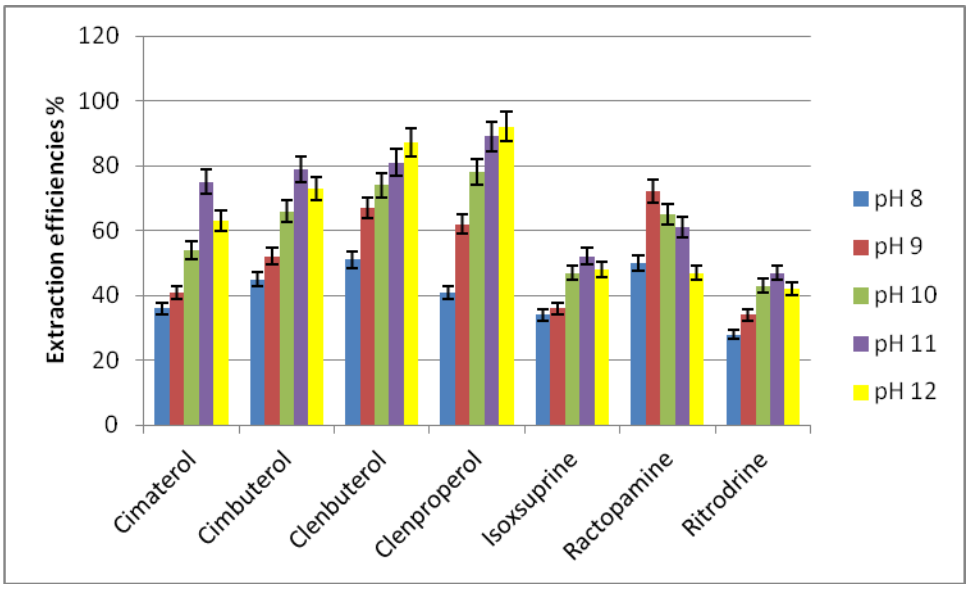


Figure 4.3.3. pH optimization of DLLME for beta-agonists in bovine muscle.

4.3.4 Optimization of disperser solvent volume

The volume of the disperser solvent plays an important role in the extraction of the analyte. Its variation has direct implications on the sedimented phase volume. At low volumes of disperser solvent, the dispersion of the extraction solvent is poor and the emulsion formation is insufficient.

The effect of the volume of acetonitrile on the extraction efficiency was studied, and 500- 2500 µL was selected. The extraction efficiencies of the analytes in acetonitrile increased from 35-53 % at 500 µL to 73–85 % at 1000 µL. The lower extraction efficiencies observed at 500 µL were due to an insufficient formation of the cloudy state, was not formed well. However increasing the volume after 1000 µL resulted in a decrease in extraction efficiencies, from 51-72 %, 28-48% and 20 -31%, for 1500 µL, 2000 µL and 2500 µL, respectively. This was due to an increase in the solubility of analytes in aqueous phase (high affinity of analytes to aqueous phase) thus leading to decrease in extraction efficiency [118-119]. Hence 1000 µL was selected as the disperser volume for all further work.

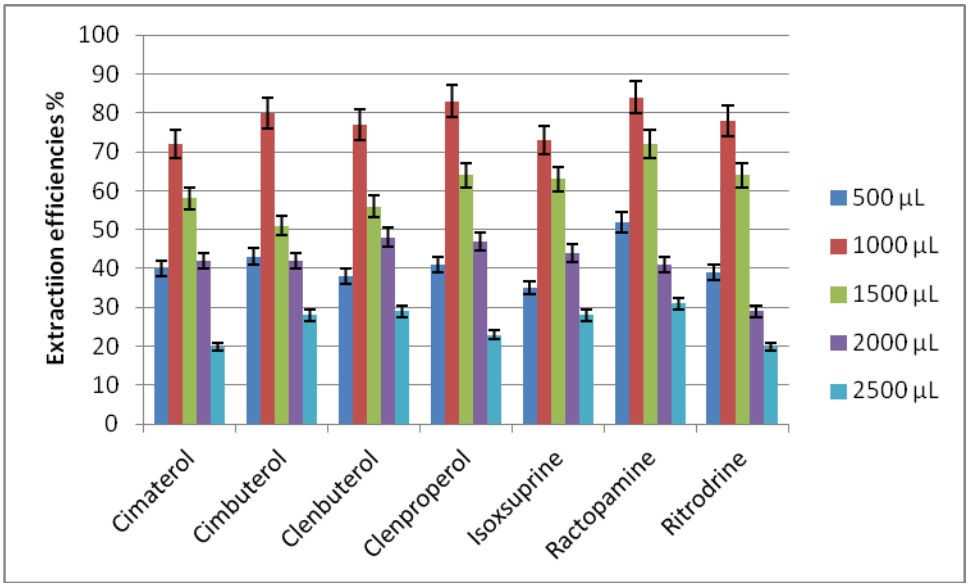


Figure 4.3.4 Disperser solvent volume optimization

4.3.5 Optimization of extraction solvent volume

The volume of extraction solvent is also very important parameter since, its variation affects the extraction efficiency and enrichment factors of analytes. In addition an increase in extraction solvent volume, increases the volume of the sedimented phase resulting in decrease of the enrichment factor [85]. Volumes of dichloromethane tested, as the extraction solvent, ranged between 50-200 µL were subjected to the same DLLME procedure. Figure 4.3.5 shows the extraction efficiencies as illustrated in figure 4.3.5 were 30-48%, 86–95%, 60–70% and 30–60% for 50 µL, 100 µL, 150 µL and 200 µL, respectively. The optimum volume for the extraction was found to be 100 µL. Volumes above 100 µL resulted in decreasing extraction effeciencies. A volume of 100 µL of dichloromethane was therefore used for all further extractions.

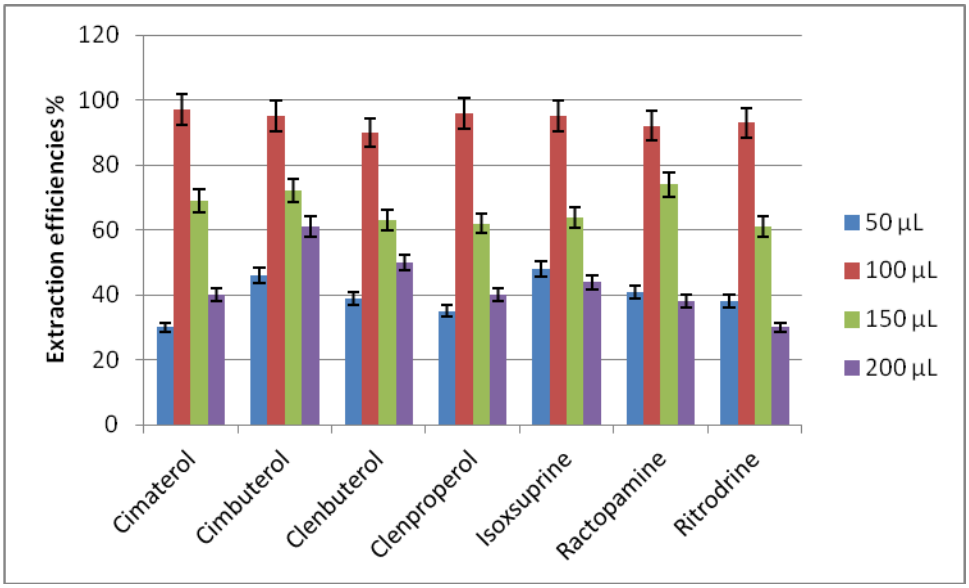


Figure 4.3.5 Extraction solvent volume optimization

4.4 DLLME method validation

The European Union (EU) Commission Decision 2002/657/EC Directive, that defines performance criteria for validation of analytical methods were used for DLLME method validation [26]. Several validation parameters such as precision, limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα), detection capability (CCβ), linearity, and recovery were determined under the optimized conditions.

Table 4.4.1: Summary of limit of detection (LOD) and limit of quantification (LOQ)

Analyte	R^2	LOD (µg kg^{-1}) n = 8	LOQ (µg kg^{-1}) n = 8
Cimaterol	0.9942	0.0738	0.246
Cimbuterol	0.9952	0.0738	0.246
Clenbuterol	0.9930	0.0781	0.260
Clenproperol	0.9948	0.0756	0.252
Isoxsuprine	0.9917	0.0922	0.307
Ractopamine	0.9915	0.0884	0.295
Ritodrine	0.9756	0.0728	0.243

Limit of detection was calculated as LOD = 3.3*(s_y/*m*) and limit of quantification as LOQ = 10*(s_y/*m*) where ***m*** is the slope of the calibration curve and S_y is the standard error of the calibration curve given by the equation below;

$$S_y = \sqrt{\sum \frac{(Y_i - mx_i - b)^2}{n-2}}$$

Where: Y_i is the y value, X_i is the x value, ***b*** is the y intercept, ***m*** is the slope and ***n*** is number of the degrees of freedom.

The R^2 LODs and LOQs of all beta-agonists are summarized in Table 4.4. All the LODs and LOQs are below the MRL levels for these drugs and ranged from 0.0728-0.0922 and 0.243 – 0.307 respectively which makes the developed method (DLLME) more suitable for its desired goal and its application in routine analysis.

The DLLME as an extraction method was applied to bovine samples spiked with beta-agonist to validate the method. Linearity was determined using pre-extraction matrix spikes which are samples that were spiked with beta-agonists standards solutions prior to extraction. It was very important to use matrix calibration standards to minimize matrix effects and improve the method precision. Linearity in the range of 0.5 – 2.0 µg kg^{-1} was obtained with regression coefficients indicated in Table 4.4.1.

For precision batches, four controls for each analytical batch were prepared. Each spiking level had seven replicates except for the blank (a batch). Bovine muscle samples were spiked at 0.5 x MRL (spike 1), 1 x MRL (spike 2), 1.5 MRL (spike 3) and a blank sample. The experiment was repeated on two occasions. The spiked bovine muscle samples after processing through DLLME sample, were analyzed by +ESI LC-MS/MS in MRM scan mode. The data obtained was then used to calculate the validation parameters i.e. limit of detection (LOD), limit of quantification (LOQ), CCα and CCβ.

Table 4.4.2. Accuracy and precision of the DLLME

Analyte	Spiking Level (ug/Kg)	Mean (n= 21) recovery (%)	Maximum Recovery (%)	Minimum Recovery (%)	CV (%)
Cimaterol	0.50 0.75 1.0	93 98 100	102 107 108	86 90 93	5 7 5
Cimbuterol	0.50 0.75 1.0	95 96 96	104 105 107	84 87 86	6 7 8
Clenbuterol	0.50 0.75 1.0	94 96 91	100 107 95	83 84 86	5 8 3
Clenproperol	0.50 0.75 1.0	93 99 91	105 108 98	84 86 86	6 7 4
Isoxsuprine	0.50 0.75 1.0	92 92 93	98 104 103	84 84 87	5 8 6
Ractopamine	0.50 0.75 1.0	92 95 91	100 104 101	82 88 84	5 6 6
Ritodrine	0.50 0.75 1.0	91 89 92	99 99 104	81 85 86	5 5 7

The validation results shown in Tables 4.4.2. and 4.4.3 The mean recoveries of spiked blank muscle samples at three levels (i.e. 0.5, 0.75 and 1.0 µg kg^{-1}) ranged from 85 to 108% and the reproducibility was between 2 and 7. The recoveries were satisfactory as they are within the acceptable range of 80 – 110% and the method is reproducible (% CV are less than 23) according to the EU Commission Decision 2002/657/EC Directive.

The method performance characteristics (decision limit and detection capacity) were also determined. The decision is defined as the limit above which it can be concluded, with an error probability of α, that a sample is non-compliant (higher than the MRL), and detection capability is defined as the smallest content of the substance that may be detected, identified and quantified in a sample with an error probability of β. The decision limit, CCα was calculated as the mean measured concentration at MRL +1.64x in-house reproducibility at this concentration and the detection capacity, CCβ was calculated as CCα+1.64 x the in-house reproducibility at MRL. The obtained CCα ranged from 0.492 to 0.559 µg kg^{-1} while CCβ ranged from 0.509 to 0.615 µg kg^{-1}.

Table 4.4.3: The validated parameters for the DLLME method

Analytes	Cimaterol	Cimbuterol	Clenbuterol	Clenproperol	Isoxsuprine	Ractopamine	Ritodrine
Spiked at 0.5 ug/kg	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mean (n= 21) recovery (%)	93	95	94	93	92	92	91
Maximum Recovery (%)	102	104	100	105	98	100	99
Minimum Recovery (%)	86	84	83	84	84	82	81
CV (%)	5	6	5	6	5	5	5
Mean conc. At MRL	0.521	0.474	0.464	0.467	0.459	0.460	0.456
Reproducibility at MRL	0.022	0.011	0.031	0.028	0.022	0.047	0.0252
CCα ($\mu g\ kg^{-1}$)	0.559	0.492	0.515	0.513	0.495	0.537	0.498
CCβ ($\mu g\ kg^{-1}$)	0.596	0.509	0.565	0.560	0.531	0.615	0.539
LOD ($\mu g\ kg^{-1}$)	0.0738	0.0738	0.0781	0.0756	0.0922	0.0884	0.0728
LOQ ($\mu g\ kg^{-1}$)	0.246	0.246	0.260	0.252	0.307	0.295	0.243
R^2	0.9942	0.9952	0.9930	0.9948	0.9917	0.9915	0.9756

4.4.1.1 Comparison of DLLME and dSPE

Sample preparation for determination of beta-agonists in bovine muscle by DLLME method was compared to dispersive solid phase extraction clean up method for the latter (Table 4.4.4).

The recoveries from spiked bovine muscle for both DLLME and dSPE are given in Table 4.4.4. The mean extraction recoveries for DLLME obtained were between 85 to 100% whereas that of dSPE was found to be between 77 to 91%. A paired t-test was performed for the two methods. For four out of the seven beta-agonists, DLLME performed better than dSPE, and there was no significant difference for the remaining three compounds. Considering the amount (large volumes on comparative basis) of organic solvents used for dSPE technique, DLLME has demonstrated to be a better sample preparation technique with respect to environmental friendliness since less of organic solvents were used, (in microliters volumes). The extraction time in DLLME is shorter and this method does not involve any labor-intensive and time consuming steps.

Table 4.4.4. The recoveries of DLLME and dSPE

Analyte	Amount added Spiked (µg kg^{-1})	N	Mean (% recovery) DLLME	Mean (% recovery) dSPE	CV (%) DLLME	CV (%) SPE
Cimaterol	0.50	7	99	83	6	7
	0.75	7	98	89	7	7
	1.00	7	100	85	5	7
Cimbuterol	0.50	7	92	84	6	7
	0.75	7	96	84	7	10
	1.00	7	96	91	8	9
Clenproperol	0.50	7	96	91	6	9
	0.75	7	99	91	7	6
	1.00	7	91	85	4	11
Clenbuterol	0.50	7	89	88	6	11
	0.75	7	96	81	8	4
	1.00	7	91	82	3	11
Isoxsuprine	0.50	7	93	89	8	4
	0.75	7	92	87	8	8
	1.00	7	93	86	6	8
Ractopamine	0.50	7	90	93	10	9
	0.75	7	95	89	6	8
	1.00	7	91	77	6	3
Ritodrine	0.50	7	91	84	8	7
	0.75	7	85	85	5	7

Analyte	Amount added Spiked (µg kg^{-1})	N	Mean (% recovery) DLLME	Mean (% recovery) dSPE	CV (%) DLLME	CV (%) SPE
	1.00	7	94	82	7	6

N = number of replicates

SD = standard deviation of 7 replicates

CV = (SD/mean)*100

4.4.2 Application of the validated DLLME method in bovine muscle

The optimized and validated DLLME method was used to analyze 26 bovine muscle samples. In 21 out of 26 samples, none of beta-agonists compounds were detected whereas, in 5 samples, clenbuterol (0.344 µg kg^{-1}, 0.421 µg kg^{-1}), Isoxsuprine (0.249 µg kg^{-1}, 0.288 µg kg^{-1}) and Ractopamine (0.582 µg kg^{-1}) clenbuterol were detected and quantified. This means there was illegal dosage of these drugs in meat producing animal, in Botswana, despite the substances being banned.

Table 4.4.5 Bovine muscle samples analysed by the validated DLLME method.

Analytes	Cimaterol	Cimbuterol	Clenbuterol	Clenproperol	Isoxsuprine	Ractopamine	Ritodrine
Spiked at 0.5 µg kg^{-1} (3 spikes)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Spike 1 recovery (%)	96	93	87	86	89	98	94
Spike 2 recovery (%)	87	84	90	85	95	103	91
Spike 3 Recovery (%)	83	89	87	94	91	88	97
Sample 1	-	-	0.344	-	-	-	-
Sample 2	-	-	-	-	-	-	-
Sample 3	-	-	-	-	0.249	-	-
Sample 4	-	-	-	-	-	-	-
Sample 5	-	-	-	-	-	-	-
Sample 6	-	-	-	-	0.288		-
Sample 7	-	-	-	-	-	-	-
Sample 8	-	-	-	-	-	0.582	-
Sample 9	-	-	-	-	-	-	-
Sample 10	-	-	0.421	-	-	-	-

CHAPTER FIVE

5.0 Conclusions

The objectives of the research were achieved with the following specific objectives completed: The mass spectrometry method was successfully developed and optimized for the determination of beta-agonists. The separation method was also successfully developed for seven beta-agonists compounds in bovine muscle. Dispersive liquid-liquid microextraction (DLLME) was also successfully developed, optimized and validated for the extraction of seven beta-agonists (Cimaterol, Cimbuterol, Clenproperol, Clenbuterol, Ractopamine, Isoxsuprine and Ritodrine) in bovine muscle using a Applied Biosystem 4000 Qtrap mass spectrometer for the simultaneous detection and identification in single analysis.

Validation of the optimized DLLME method was done according to the Commission Decision 2002/657/EC and ISO 17025. The validated DLLME method was then applied on real samples (bovine muscle). An environmentally friendly sample preparation method (DLLME) for simultaneous determination of seven beta-agonists in bovine muscle by +ESI-LC-MS/MS was developed. This method is simple, cheap and it is short. The sensitivity of this method was proven, since it is capable of testing at levels below the appropriate Minimum Performance Residue Limits (MPRLs) for the beta-agonists (0.5 µg kg^{-1}).The method was applied to 26 bovine muscle samples collected from two Botswana Meat Commission abattoirs (BMCs).

In 21 of the 26 samples, no beta-agonists were found. In the other 5 samples, clenbuterol was detected in two samples (one sample gave 0.344 µg $kg^{-1}$and

another gave 0.421 µg kg^{-1}). Also Isoxsuprine was detected in two samples (one sample gave 0.249 µg kg^{-1}while another gave 0.288 µg kg^{-1}) and Ractopamine (0.582 µg kg^{-1}) were detected and quantified. The implication is that some of these drugs are being used by some Botswana farmers for medicinal purposes.

The analytical method (optimized DLLME) in the present study was validated using ISO 17025 and the EU criteria (Commission Decision 2002/657/EC). Good precision, repeatability and recoveries were obtained. The limits of detection and quantification for the residues were between 0.0792 – 0.122 µg $kg^{-1}$and 0.264 – 0.408 µg kg^{-1}respectively for the seven beta-agonists. The overall recoveries were between 85% and 100% with the relative standard deviations (RSDs) between 3.0% and 10%. The recoveries from dispersive liquid-liquid micro-extraction (DLLME), 85% to 100% were comparable to those of dSPE (77% to 93%). Also the Decision limit (CC_α) and Detection capability (CC_α) were calculated. For cimaterol, [(CC_α) was 0.559, above this level the sample is non-compliant and (CC_β) was 0.596 being detection capability]. The DLLME method was used to test bovine muscle samples from BMC, and low concentrations of some of beta-agonists were accurately determined showing the sensitivity of this method.

5.2 Recommendation

The recommendation for future research on this subject, the limit of detection (LOD) and limit of quantification (LOQ) of analysis for beta-agonists in bovine muscle by DLLME can be improved by the use of surfactants, since most cases the muscle samples have some fats, which give a problem. This method can be used in routine laboratory analysis of beta-agonists for surveillance purposes. More sampling from

Botswana slaughter houses is recommended since some traces of these prohibited drugs were detected.

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